

Bioinformatics Analysis of Four Gallinacin Genes for Immunity Traits in Chickens

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Abstract

Four candidate gallinacin polymorphic genes were sequenced in one direction from Fayoumi (F), Rhode Island Red (R) and their crosses ($\frac{1}{2}R\frac{1}{2}F$ and $\frac{1}{2}F\frac{1}{2}R$). Pairwise bioinformatics sequencing of *GAL* genes of the parents relative to F₁ generation was identified. Bioinformatics analyses of *GAL* genes showed many sequence variations. The pairwise sequence alignment between F and $\frac{1}{2}F\frac{1}{2}R$ were identified, being 17 SNPs with identity ratio of 97% for *GAL 2* gene, many SNPs and gaps with low identity ratio of 50% for *GAL 3* gene, ten SNPs with identity ratio of 98% for *GAL 4* gene and 18 SNPs with identity ratio of 97% for *GAL 5* gene. Between F and $\frac{1}{2}R\frac{1}{2}F$ there were 11 SNPs with identity ratio of 98% for *GAL 2* gene, 15 SNPs with identity ratio of 98% for *GAL 3* gene, 20 SNPs with identity ratio of 96% for *GAL 4* gene and many SNPs with low identity ratio of 50% for *GAL 5* gene. Between R and $\frac{1}{2}F\frac{1}{2}R$, the sequence in *GAL 2* gene were 12 SNPs with identity ratio of 98%, 38 SNPs with identity ratio of 94% in *GAL 3* gene, many SNPs and gaps with low identity ratio of 50% in *GAL 4* gene and 37 SNP with identity ratio of 90% in *GAL 5* gene. The sequence between R and $\frac{1}{2}R\frac{1}{2}F$ being 21 SNPs with identity ratio of 96% for *GAL 2* gene and many SNPs and gaps with low identity ratio of 50% for *GAL 3* gene. The gene SNP-trait associations are not identical among all genes in the gallinacins cluster. Bioinformatics analyses of *GAL* genes showed many sequence variations and mutations in intronic regions. The SNPs identified in gallinacin genes could be used in marker assisted selection in breeding program to enhance immune responses to *Salmonella* disease in chickens.

Keywords: Chickens, Bioinformatics, Gallinacin genes, *Salmonella*, Immune response.

Introduction

In recent years, advances in molecular technology have created a new horizon for the genetic improvement of disease-resistant traits in poultry. Detection of associations between candidate genes or markers and *Salmonella* bacterial burden could also lead to improve disease resistance in chickens (Rothschild, 1997). The identification of direct or indirect molecular markers for these traits would facilitate the use of these markers in selection or in gene introgression (Wakchaure *et al.*, 2015). However, *Salmonella typhimurium* and *Salmonella enteritidis* are acute systemic diseases in young chicks (Friedman *et al.* 2003). In this concept, few reports on *Salmonella* serovars distribution in broiler farms were documented (Ammar *et al.*, 2009; Abd El-Ghany *et al.*, 2012; El-Safey, 2013). Beta-defensins belong to a family of defensins which is ataxonomic group of cationic antimicrobial peptides (AMPs) with broad-spectrum antimicrobial activity against various microorganisms, fungi, and viruses (Lai and Gallo, 2009). Gallinacins are cysteine-rich antimicrobial peptides characterized by six cysteine residues, which form three pairs of disulfide bridges (Sugiarto and Yu 2004). These are relatively small antimicrobial peptides, typically less than 100 amino acids in size, which possess a broad range of antimicrobial activity (Sugiarto and Yu 2004). Gallinacins are functional analogues of the mammalian beta-defensins and play an important role in the innate immunity against microbial infections in chickens. In chickens, gallinacin genes 1 to 13 have been mapped within an

86-kb region of chromosome 3 and each gallinacin gene possesses the same genomic structure of four short exons that are separated by three introns of various lengths (Xiao *et al.*, 2004). These gallinacin genes are abundant in cells that are involved in the innate immune system response against microbial infections (Ganz, 2003; Xiao *et al.*, 2004; Hasenstein *et al.*, 2006; Hasenstein and Lamont, 2007; Morammazi and Habibi, 2017) and they exhibit wider range of antimicrobial activity against both gram-positive and gram-negative bacteria (Higgs *et al.*, 2005). Since, planning to use genetic selection and breeding program for resistance to disease using molecular markers would result in a resistance population and reduce the price of treatment (Pruthviraj *et al.*, 2016). In breeding programs, when using popular techniques such as those relying upon to polymerase chain reaction (PCR), it is better to consider molecular markers that are close to health-related peptides' ordination. To date, some studies have been conducted to find the connection between poultry genome and poultry diseases (Kramer *et al.*, 2003). However, beta-defensin genes have been neglected. The main objective of the present study was to identify the SNPs of gallinacins genes in parental breeds of Fayoumi (F) and Rhode Island Red breed (R) and their crosses $\frac{1}{2}R\frac{1}{2}F$ and $\frac{1}{2}F\frac{1}{2}R$ using bioinformatics analysis.

Materials and methods

Breeding plan and management

Bioinformatics analysis was performed in an crossbreeding experiment involving Fayoumi (F) and Rhode Island Red (R) and their crosses ($\frac{1}{2}R\frac{1}{2}F$ and $\frac{1}{2}F\frac{1}{2}R$). The details of the crossbreeding experiment of the studied populations were described in the pervious manuscript (Saleh *et al.*, 2020).

The chicks were kept under similar hygienic and environmental conditions, vaccinated against Newcastle and Gumboro disease, housed under continuous fluorescent lighting (10 watt/m²) and provided un-medicated corn soybean-based meal diet (not containing antibiotics, coccidiostats, or growth promoters). Fresh water was provided for the chicks *ad libitum*. Chicks produced from all genetic groups were fed *ad libitum* on diet containing 21% protein and 2700 kcal/kg during the growing period (from hatch up to 10 weeks of age). Minerals and vitamins were adequately supplied to cover the chicks' requirements according to NRC (1994).

Blood sampling and DNA extraction

Ninety-six chicks belonging to four chicken genetic groups (24 chicks from each group of F, R, $\frac{1}{2}F\frac{1}{2}R$ and $\frac{1}{2}R\frac{1}{2}F$) were used in the molecular genetic analyses. The blood samples were collected from pedigreed chicks with the least relationship to decrease the genetic similarity between the genotyped chicks. Approximately 3-5 ml venous blood sample per chick was collected from the wing vein by a 2-gauge 1.5-injection needle into tubes containing

EDTA as anticoagulant. Genomic DNA was extracted from Whole Blood Genomic DNA Purification Mini Kit (Cat No. #K0781, Thermo Scientific). The laboratorial analyses for molecular biology were carried out in the Labs of Genetics Department, Faculty of Agriculture, Benha University, Egypt, and Avian Pathology Section, Department of Veterinary Medicine, University of Bari, Italy since April to September 2017.

PCR amplification

Preliminarily, the primer sequences were assessed *insilico* (<http://insilico.ehu.es/PCR/>) and each gene of *Gal 2*, *Gal 3*, *Gal 4* and *Gal 5* (each coding for a respective avian beta-defensin) was characterized by amplifying a portion using the proper primer pair listed in Table 1 (Hasenstein *et al.*, 2006; Saleh, 2019). On chromosome 3, PCR amplifications were carried out in 50 μ l reaction mixture composed of 4 μ l genomic DNA (100 ng/ μ l) as a template, 50 pmol of each primer, 2.5 mM dNTP' (dATP, dCTP, dTTP and dGTP; ABgene, Surrey, UK), 10X PCR buffer, 25 mM MgCl₂, and 2.5 unit *Taq* DNA polymerase. After electrophoresis, the gel was stained with ethidium bromide 0.5 μ g/ml. Gallinacin genes fragments were visualized by using a UV transilluminator and documented in Gel DocTMXR⁺ (BIO-RAD). The PCR products were purified using QIAquick PCR purification kit (Qiagen, Inc.) and the purified products were used in the subsequent sequencing reactions.

Table 1 Primer sequence of Gal genes in chromosome 3

Gene	Primer sequences (forward/reverse)	PCR Product size (bp)	GenBank accession No.
<i>Gal 2</i>	5'-GGCACAAAGGGTAAAGTATGG -3' 5'-GAGGGGTCTTCTTGCTGCTGA -3'	583	(AY621317)
<i>Gal 3</i>	5'-GCACCACAAGAAGCCCAGGAA -3' 5'-AACTCCAGCCCTTACCACTCA -3'	664	(AY621318)
<i>Gal 4</i>	5'-TGGGGATCTTAGAGGTCTTTT -3' 5'-TTTTCCACAGATATTGCTTTT -3'	600	(AY621319)
<i>Gal 5</i>	5'-CTCCCAGCAAGAAAGGAACCTG -3' 5'-CACAGTCTGGGGTAATCCTCG -3'	623	(AY621320)

Sequencing of GAL genes

The purified PCR fragment was ligated in pGEMR-T Easy Vector Systems (Promega, Cat. #: A1360) according to the manufacturer's instructions. The purified plasmids were examined and sequenced using electrophoresis on 1.5% agarose gel using GeneRulerTM 1 kb DNA Ladder (Cat. #: SM0313) of Macrogen Company (South Korea) to confirm the recombinant plasmids. The obtained sequences for *GAL 2*, *GAL 3*, *GAL 4* and *GAL 5* genes were examined for vector contamination using the VecScreen tool (<http://www.ncbi.nlm.nih.gov/tools/vecsreen>). While Jalview software was used to show single nucleotide polymorphisms (SNPs) resulted from the alignment of obtained sequences (<http://www.jalview.org/>). The

obtained sequences were submitted in GenBank under the accession numbers.

Bioinformatics sequencing and pairwise alignment of GALlinacin genes

PCR products of *GAL 2* (583 bp), *GAL 3* (664 bp), *GAL 4* (600 bp) and *GAL 5* (623 bp) genes were partially sequenced and registered for the genetic groups of F, R, $\frac{1}{2}F\frac{1}{2}R$ and $\frac{1}{2}R\frac{1}{2}F$ in the NCBI database under the following accession numbers: MH000270.1, MH000271.1, MH000272.1 and MH000273.1 for *GAL 2* gene; MH000274.1, MH000275.1, MH000276.1 and MH000277.1 for *GAL 3* gene; MH000278.1, MH000279.1, MH000280.1 and MH000281.1 for *GAL 4* gene; MH000282.1, MH000283.1, MH000284.1 and MH000285.1 for *GAL 5* gene. The VecScreen

database analysis, revealed no contamination with vector sequences. Assessment of the genetic variability in the studied populations was performed via identifying SNPs and gaps in *GAL 2*, *GAL 3*, *GAL 4* and *GAL 5* genes in terms of sequences of F₁ chicken and the parental sequences.

Results and discussion

Pairwise sequence of *GAL* genes of the parents relative to F₁ generation

The pairwise sequence alignment of *GAL* genes of the parents compared with F₁ generation is illustrated in Table 2 and Figures 1, 2, 3 and 4. The sequence variations of *GAL* genes revealed that the genetic backgrounds in the studied genetic groups of chickens are highly polymorphic.

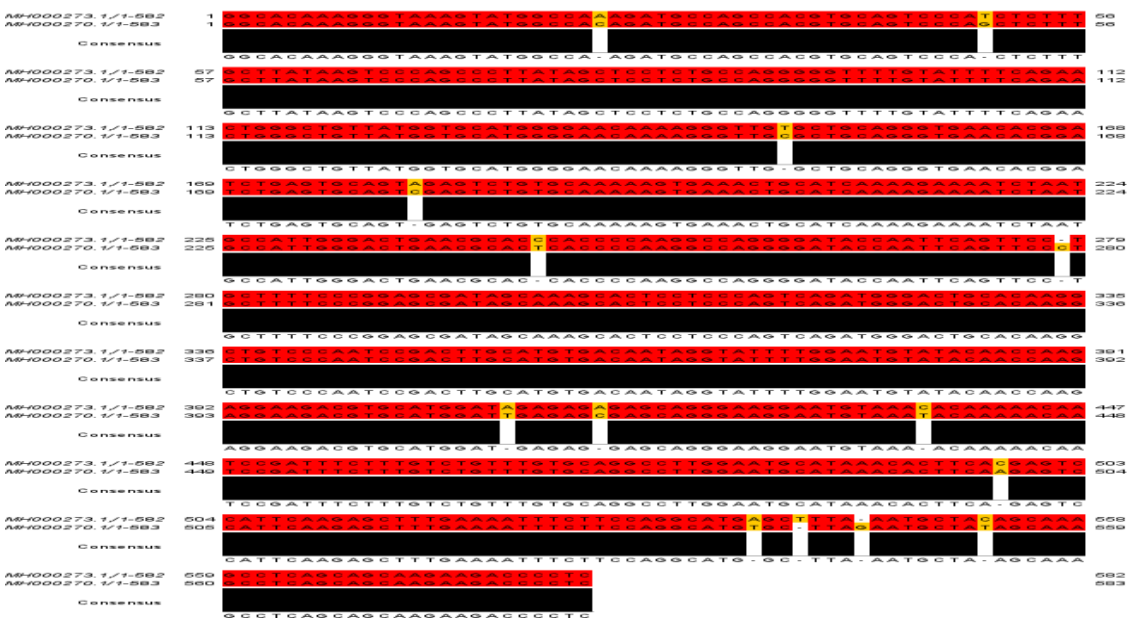
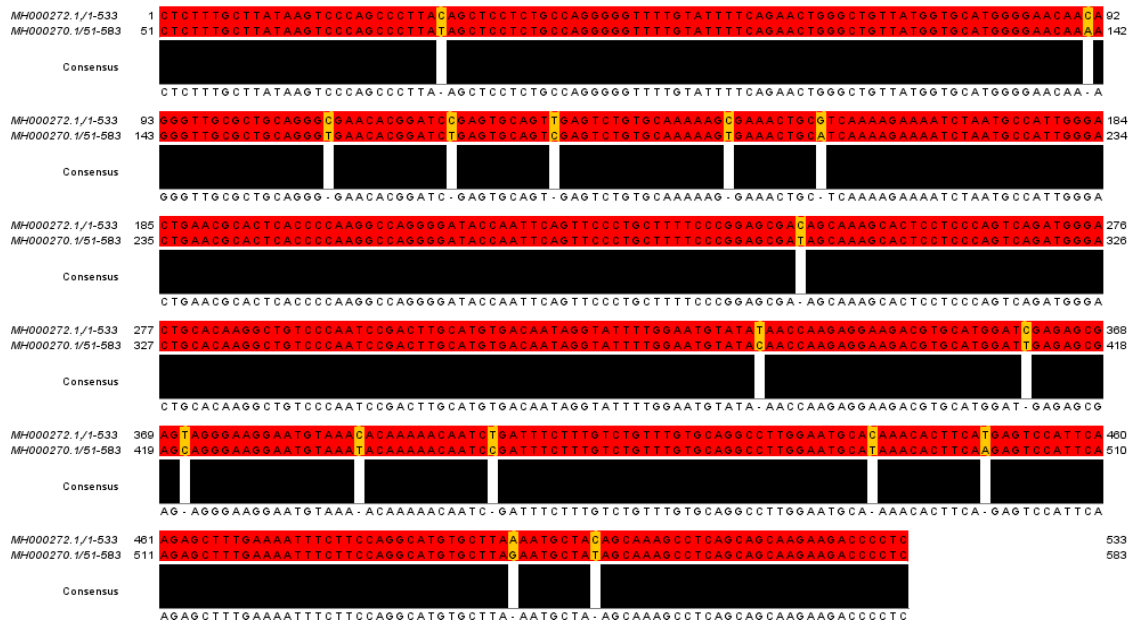
Table 2 Pairwise sequence alignment of *GAL* genes of the parents compared with F₁ generation

Gene	Pairwise genetic groups	No. of SNPs	No. of gaps	Identity ratio (%)
<i>GAL 2</i>	F with ½F½R	17	-	97
	F with ½R½F	11	3	98
	R with ½F½R	12	-	98
	R with ½R½F	21	1	96
<i>GAL 3</i>	F with ½F½R	Many	Many	50
	F with ½R½F	15	-	98
	R with ½F½R	38	1	94
	R with ½R½F	Many	Many	50
<i>GAL 4</i>	F with ½F½R	10	1	98
	F with ½R½F	20	-	96
	R with ½F½R	Many	Many	50
	R with ½R½F	Many	Many	50
<i>GAL 5</i>	F with ½F½R	18	-	97
	F with ½R½F	Many	Many	50
	R with ½F½R	Many	Many	50
	R with ½R½F	37	2	90

These high genetic variations were located in the regions associated with the innate immune response to caecal bacteria (Sugiarto and Yu, 2004; Higgs *et al.*, 2005; Morammazi and Habibi, 2017) and having the role in increasing the resistance to diseases (Bar-Shira and Friedman, 2006). It is essential to consider further research in the local poultry resources because these indigenous populations can have valuable genes pool or major supply of genes resistant to the local harsh climate, i.e. the genetic groups studied could be

considered in poultry breeding programs of genetic selection especially for disease resistance.

In terms of gallinacin 2 genomic DNA sequence, a 583 bp product was amplified and 17 SNPs were identified between F and ½F½R with high identity ratio of 97% (Fig. 1), 11 SNPs and three gaps between F and ½R½F with high identity ratio of 98% (Fig. 2), 12 SNPs between R with ½F½R cross with also high identity ratio of 98% (Fig. 3) and 21 SNPs and one gap between R and ½R½F cross with high identity ratio of 96% (Fig. 4).



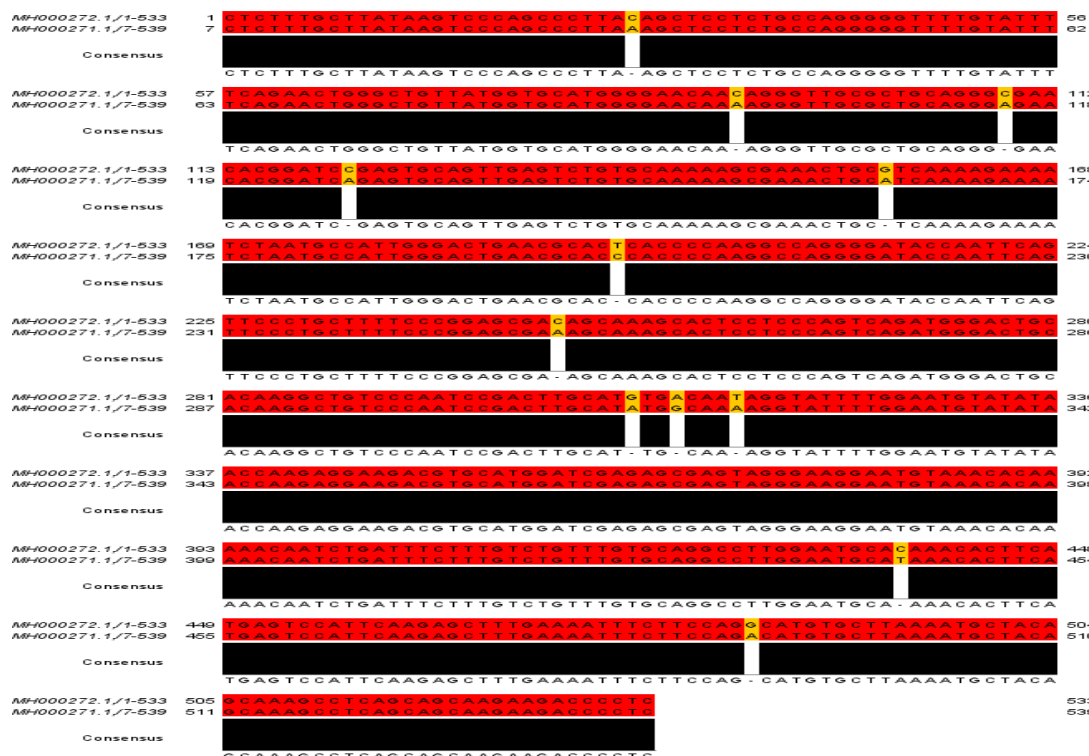


Fig. 3: Pairwise sequence alignment of GAL 2 gene between R breed and 1/2F1/2R cross.

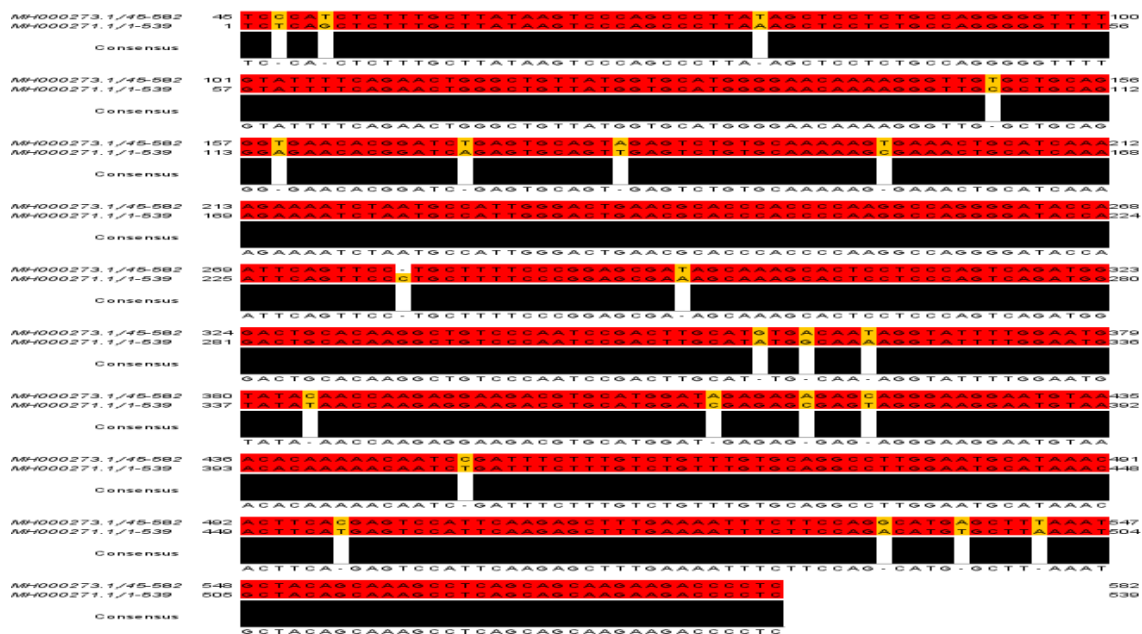
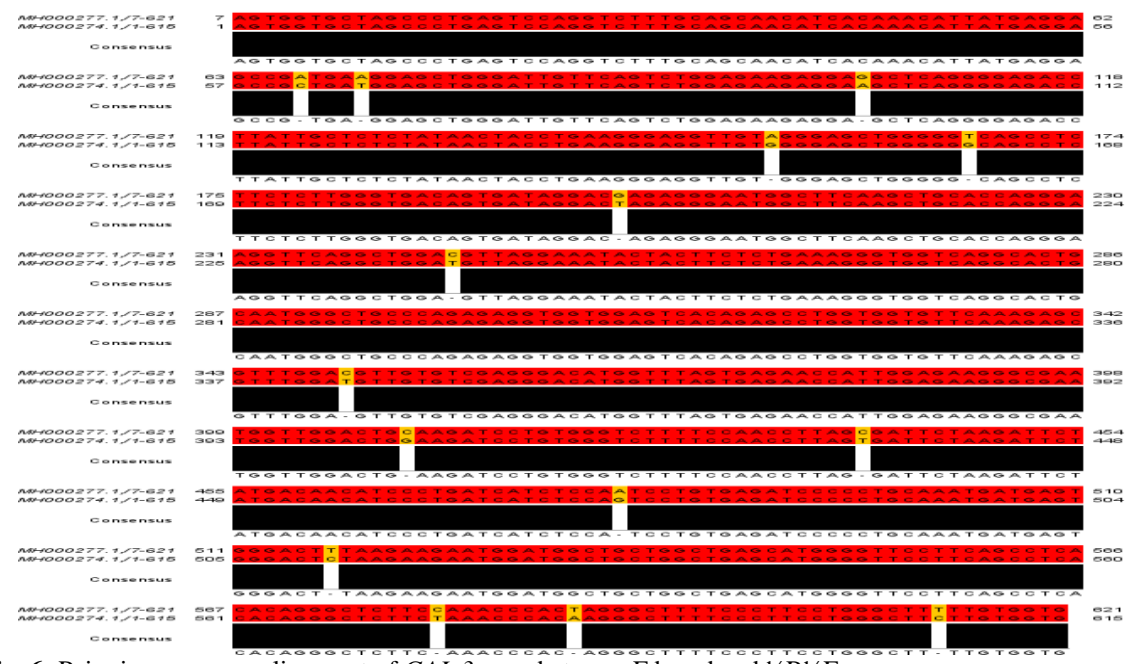
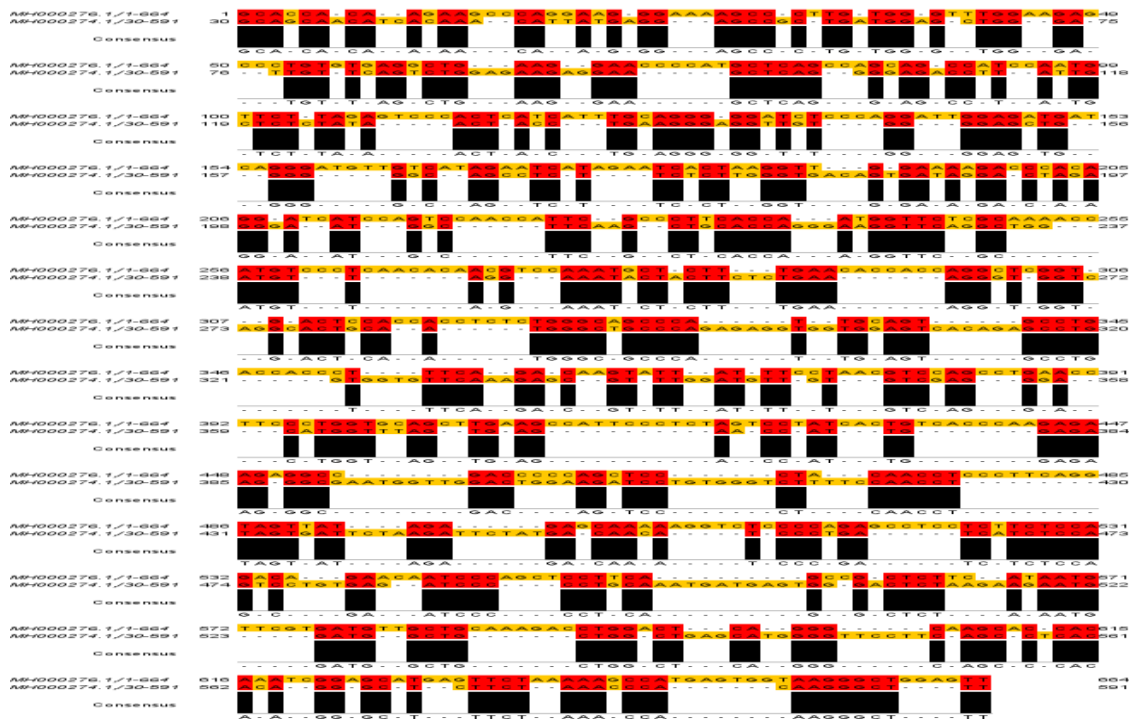


Fig. 4: Pairwise sequence alignment of GAL 2 gene between R breed and 1/2R1/2F cross.

A 664 bp product was amplified for gallinacin 3 genomic DNA sequence and many SNPs and gaps were identified between F and 1/2F1/2R with low identity ratio of 50%, while 15 SNPs were identified between F and 1/2R1/2F cross with high identity ratio of

98%. The pairwise sequence alignment of R with 1/2F1/2R cross showed that there was 38 SNPs and one gap with identity ratio of 94%, while there were lot of SNPs and gaps with low identity ratio of 50% between R and 1/2R1/2F cross (Table 2).



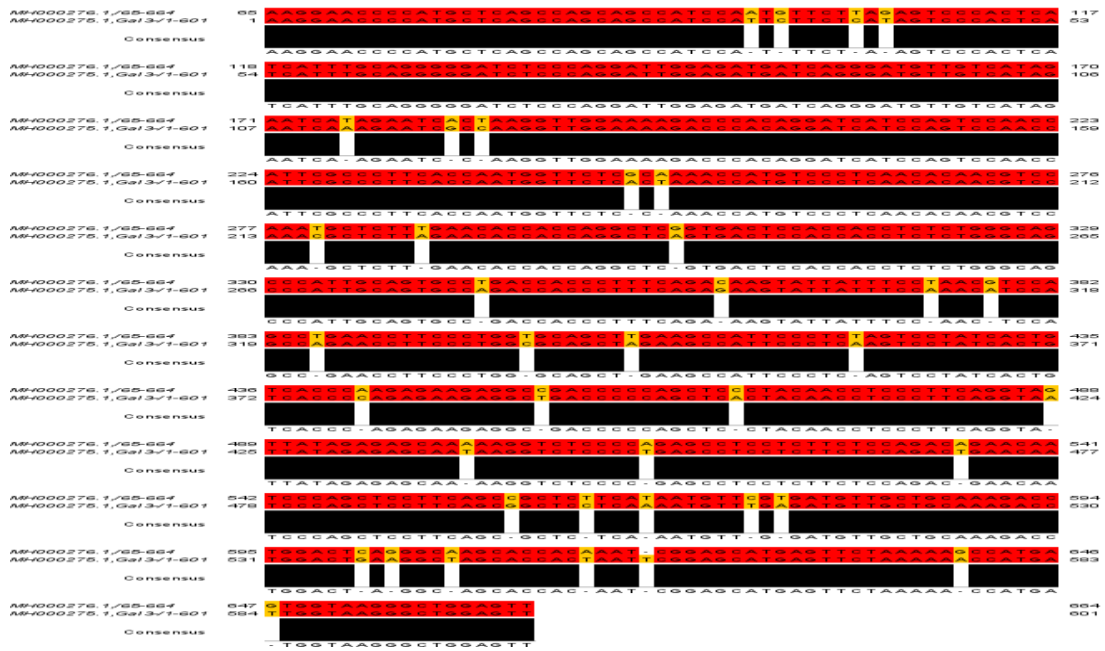


Fig. 7: Pairwise sequence alignment of *GAL 3* gene between R breed and $\frac{1}{2}F\frac{1}{2}R$ cross.

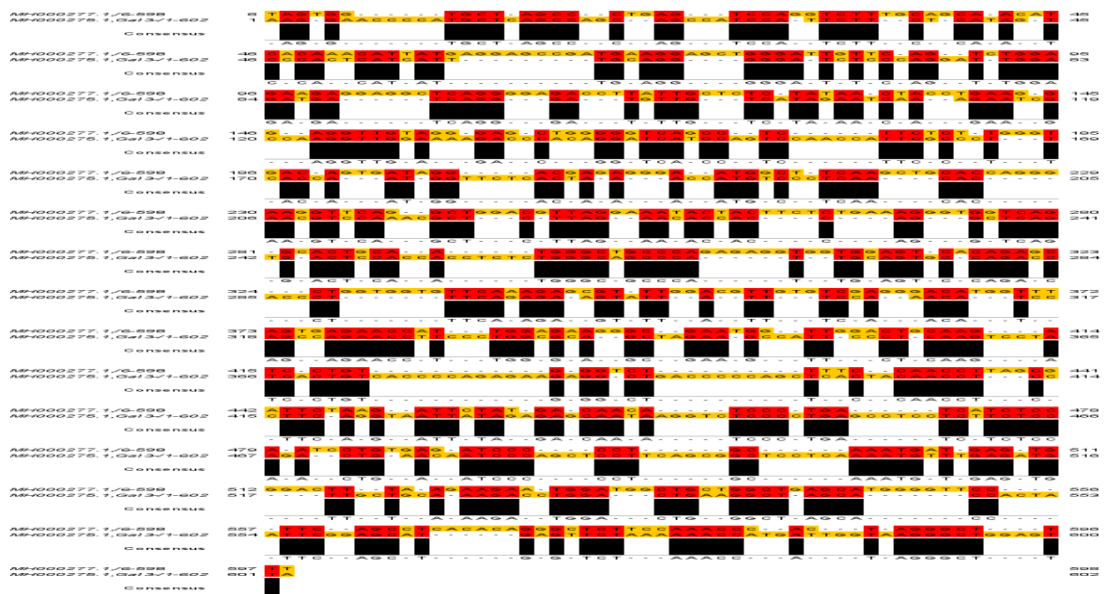


Fig. 8: Pairwise sequence alignment of *GAL 3* gene between R breed and $\frac{1}{2}R\frac{1}{2}F$ cross.

Regarding the gallinacin 4 genomic DNA, a 600 bp product was amplified and ten SNPs and one gap were identified between F and $\frac{1}{2}F\frac{1}{2}R$ cross with high identity ratio of 98% and 20 SNPs between F and

$\frac{1}{2}R\frac{1}{2}F$ cross with also high identity ratio of 96%. The pairwise sequence alignment of R with $\frac{1}{2}F\frac{1}{2}R$ or $\frac{1}{2}R\frac{1}{2}F$ crosses showed that there were many SNPs and gaps with low identity ratio of 50% (Table 2).

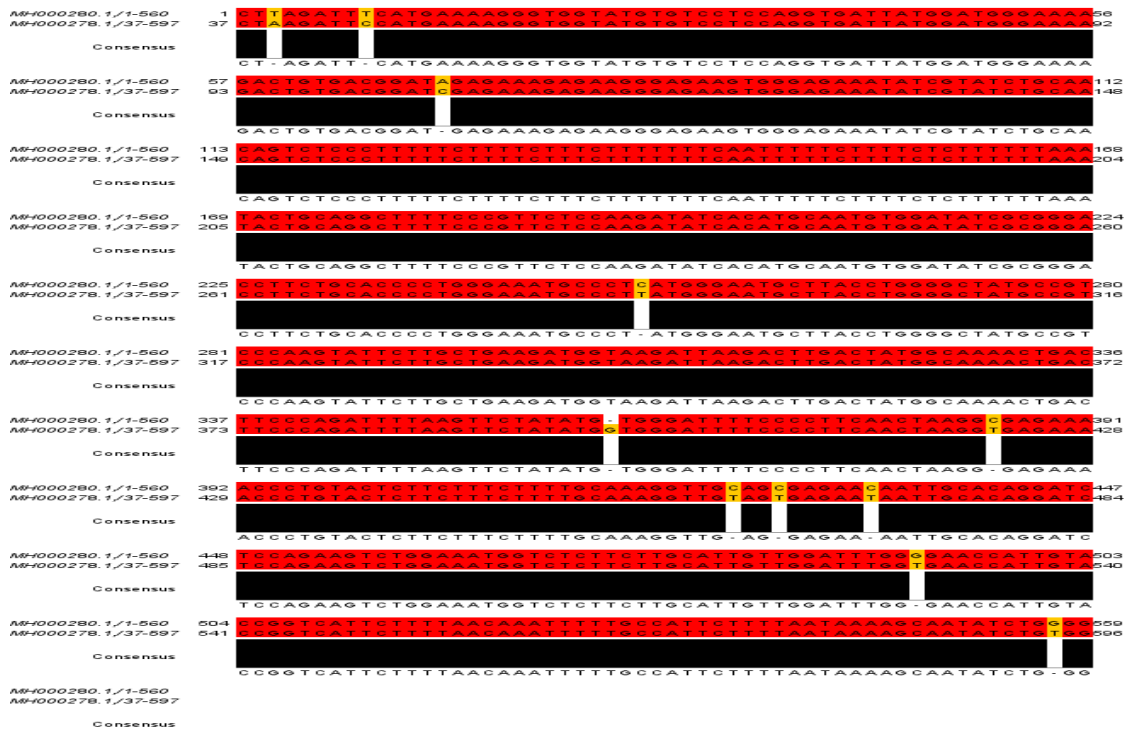


Fig. 9: Pairwise sequence alignment of *GAL 4* gene between F breed and 1/2F/2R cross.

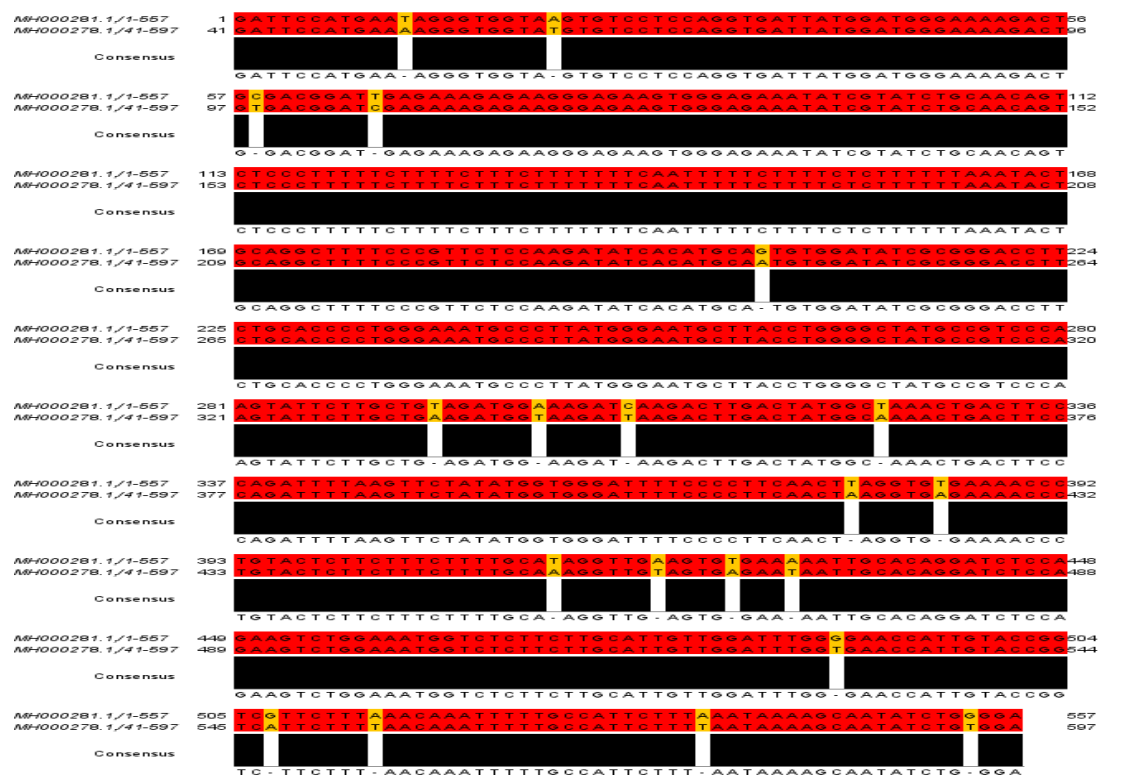


Fig. 10: Pairwise sequence alignment of *GAL 4* gene between F breed and 1/2R/2F cross.

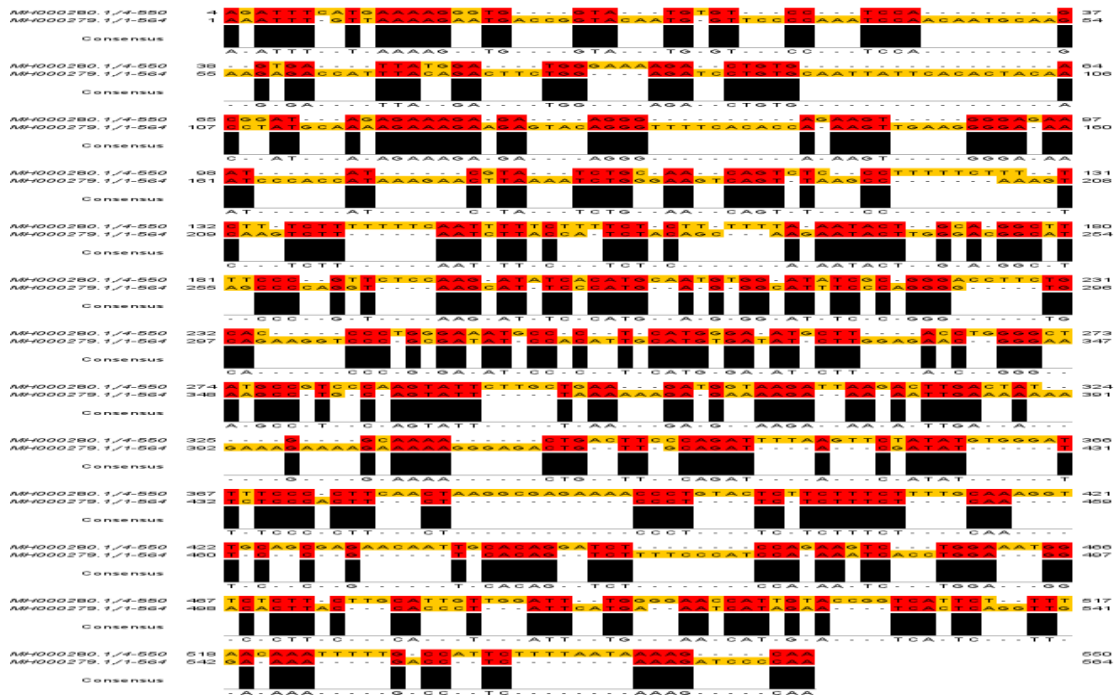


Fig.

11: Pairwise sequence alignment of *GAL 4* gene between R breed and $\frac{1}{2}F\frac{1}{2}R$ cross.

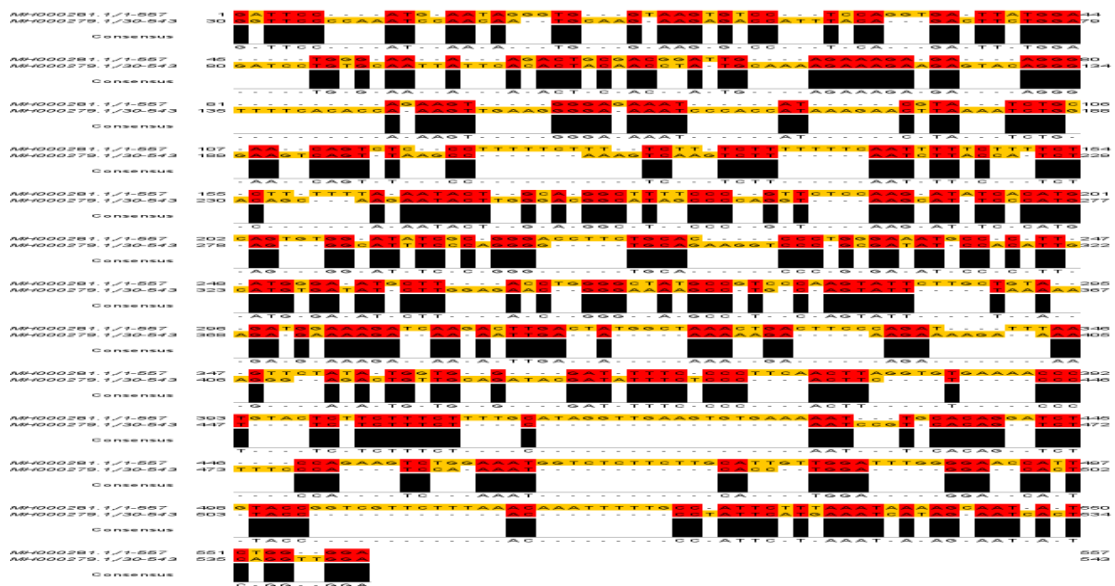


Fig. 12: Pairwise sequence alignment of *GAL 4* gene between R breed and $\frac{1}{2}R\frac{1}{2}F$ cross.

A 623 bp product was amplified from gallinacin 5 genomic DNA, showing there were 18 SNPs between F and $\frac{1}{2}F\frac{1}{2}R$ with high identity ratio of 97% and many SNPs and gaps between F and $\frac{1}{2}R\frac{1}{2}F$ cross with low identity ratio of 50%. The pairwise sequence showed also that there were 37 SNPs and two gaps between R and $\frac{1}{2}F\frac{1}{2}R$ with high identity ratio of 90% and considerable numbers of SNPs and gaps with low identity ratio of 50% between R and $\frac{1}{2}R\frac{1}{2}F$ (Table 2). Yacoub *et al.* (2011) reported that the *GAL 3*, *GAL 4*, *GAL 5* and *GAL 10* genes were polymorphic because there were many (SNPs) in line 6 resistant and line 7 susceptible to Marek's Disease of White Leghorn chickens and some of these SNPs were located in

intronic region and the rest were in exonic region. Hasenstein *et al.* (2006) found that a total of 43 SNPs were identified within the sequenced regions of *GAL 2*, *GAL 3*, *GAL 4*, *GAL 5* and *GAL 7* with an SNP rate of 13.2 SNPs/kb, while Wong *et al.* (2004) reported lower SNP rate of 5 SNPs/kb across the entire chicken genome. However, with F1 generation from a cross between divergent breeds, substantial linkage disequilibrium exists. Therefore, it is not possible to determine if the SNPs identified in the present study are causal mutations or are in linkage with a causal mutation elsewhere in the gallinacin gene cluster. The chicken genome was sequenced and annotated (Hillier *et al.*, 2004), and along with it, an SNP map

consisting of 2.8 million SNPs was created (Wong *et al.*, 2004). This makes it possible to utilize the

locations of known SNPs to fine-map a region of interest in the chicken genome.

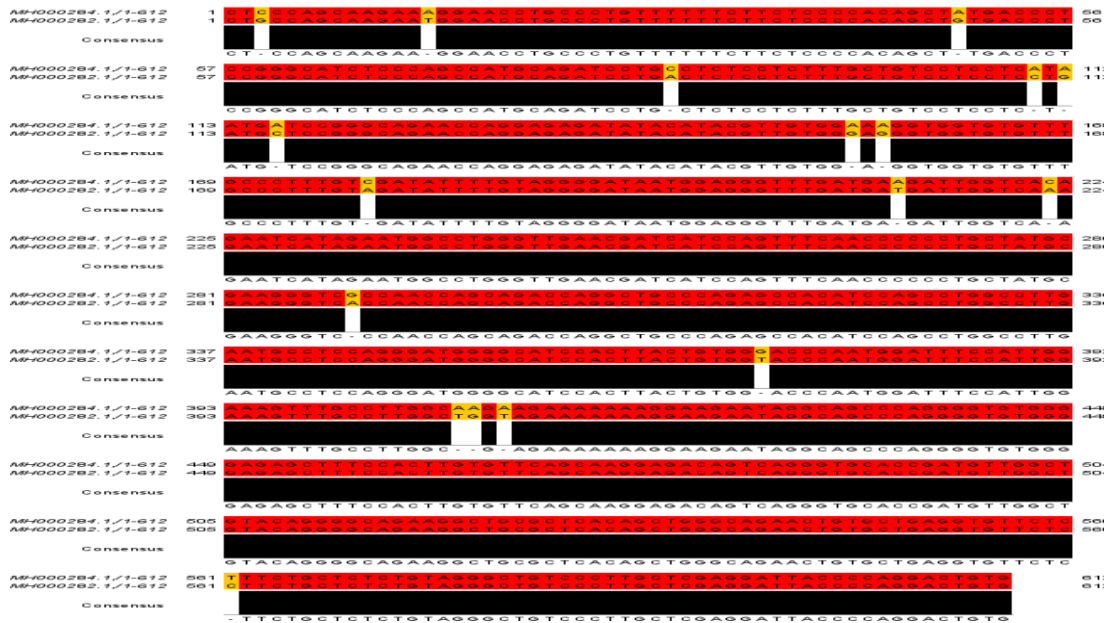


Fig.

13: Pairwise sequence alignment of GAL 5 gene between F breed and 1/2F1/2R cross.

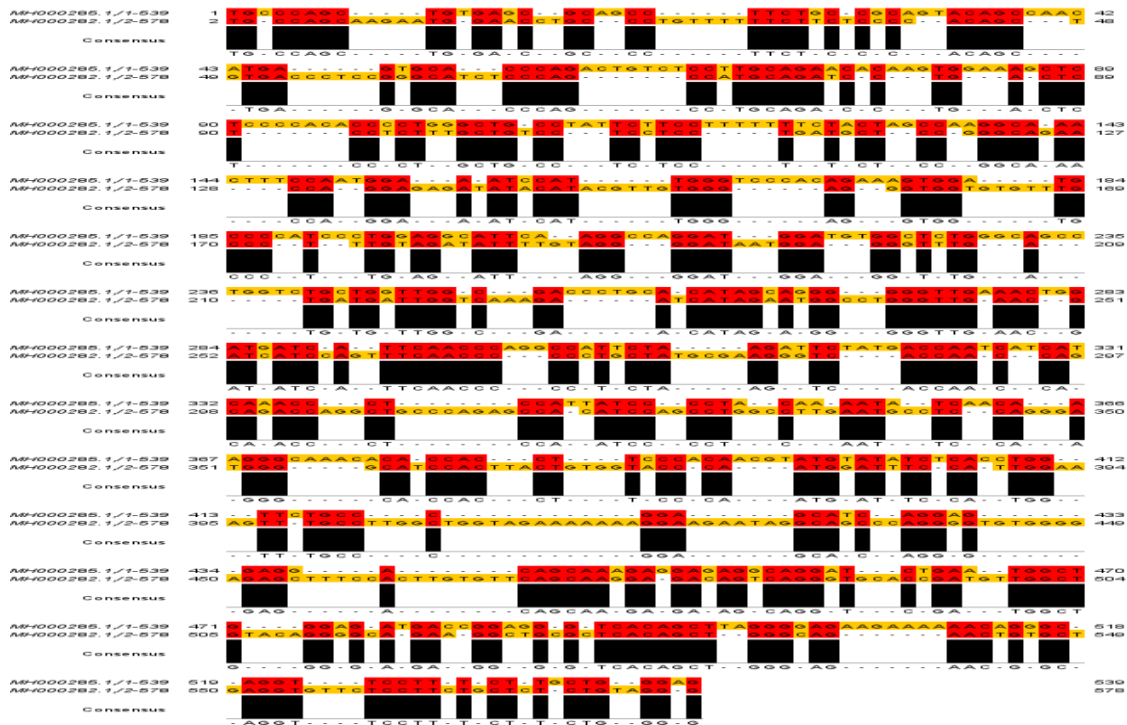


Fig. 14: Pairwise sequence alignment of GAL 5 gene between F breed and 1/2F1/2F cross.

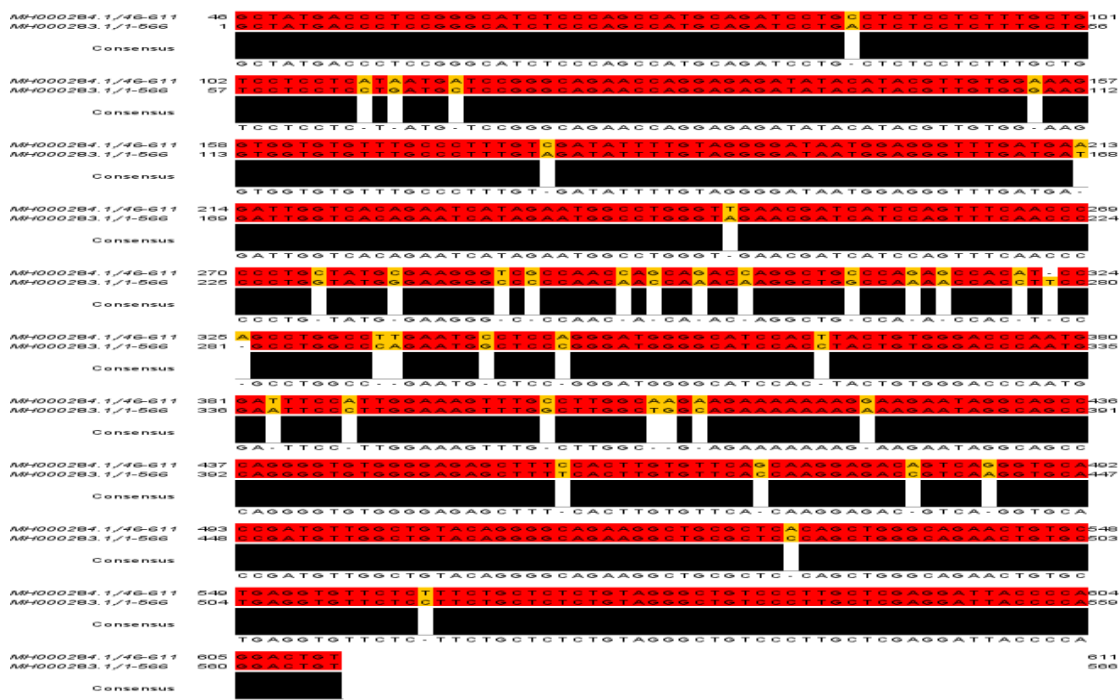


Fig. 15: Pairwise sequence alignment of *GAL 5* gene between R breed and $\frac{1}{2}F\frac{1}{2}R$ cross.

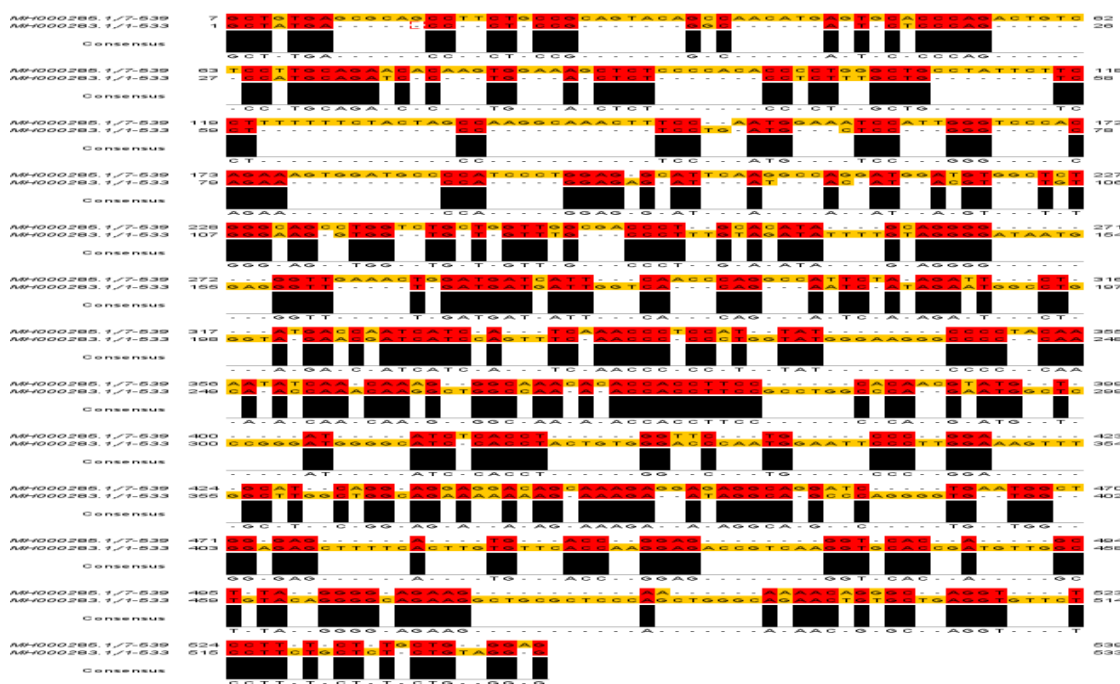


Fig. 16: Pairwise sequence alignment of *GAL 5* gene between R breed and $\frac{1}{2}R\frac{1}{2}F$ cross.

In all Figures 1 up to 4, the sequence variations for *GAL 2*, *GAL 3*, *GAL 4* and *GAL 5* genes in F, R, $\frac{1}{2}F\frac{1}{2}R$ and $\frac{1}{2}R\frac{1}{2}F$ indicate that there were many mutations or SNPs in F_1 generation and the parental breeds, i.e. the genetic variations in sequences of *GAL* genes are quite satisfactory for undertaking association studies and to know the causes of phenotypic variation and susceptibility to diseases in the studied genetic groups of chickens. Moreover, the nucleotide sequences analyzed here indicated that *GAL* genes had many

intronic mutations. Although these mutations in intronic regions did not affect the structure of protein molecules, they can provide excellent molecular markers in poultry breeding programs of genetic selection towards an increase in diseases immune responses (Morammazi and Habibi, 2017).

The high genetic variation in beta-defensin genes obtained in the present and other studies (Hasenstein *et al.*, 2006; Morammazi and Habibi, 2017) have shown considerable roles in the innate immune

response to gram-negative and gram-positive bacteria (Sugiarto and Yu, 2004; Higgs *et al.*, 2005), an increase in resistance by enhancing the recruitment of macrophages, granulocytes, and lymphocytes of the infected tissues (Bar-Shira and Friedman, 2006), and an existence of genetic associations with immune response and bacterial loads (Hasenstein *et al.*, 2006; Hasenstein and Lamont, 2007; van Dijk *et al.*, 2008). This making the sequence variations of GAL genes in the genetic groups are promising genetic markers in assisting of chickens selection programs.

Conclusions

Based on DNA sequencing, variations in GAL genes can be employed potentially in marker-assisted selection programs. Therefore, the GAL 3 and GAL 5 genes could be used as genetic markers in selection programs to improve the genetic immune response against Salmonella in chickens.

The sequence of gallinacin genes had many mutations present in intronic regions which opens up more prospects for associations and responses to pathogens studies that may help in development of a PCR genotyping test to select the parents with better immunity.

Acknowledgments

The authors would like to thank the European Union Commission, that partially funded this Joint Master Degree of TEMPUS program (Project No. 543865). The authors are gratefully acknowledge the support and help of members of Research Labs Park and Labs of Genetics Department, Faculty of Agriculture, Benha University, Egypt, and Molecular Biology Labs of Avian Pathology Section, Department of Veterinary Medicine, University of Bari, Italy.

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